THE PATENTS ACT, 1970

It is hereby certified that annexed hereto is a true copy of Application, Complete Specification & Abstract of the extract of Patent Application No.1061/CHE/2003, dated 30/12/2003 by M/s. Bharat Biotech International Limited, Genome Valley, Turkapalli, Shameerpet Mandal, Rangareddy District, Hyderabad, Andhra Pradesh, India, PIN: 500 078.

IN/04/257

.....In witness thereof

I have hereunto set my hand

Dated this the 13th day of September 2004

M.S. Venkataraman

(M.S. VENKATARAMAN)
Assistant Controller of Patents & Designs

PATENT OFFICE BRANCH
GOVERNMENT OF INDIA
Industrial Complex, 6th Floor, Annex.II
No.444, Anna Salai, Teynampet,Chennai – 600 018.

FORM 1

THE PATENTS ACT, 1970
(39 of 1970)

APPLICATION FOR GRANT OF A PATENT

(See Sections 54 ; Rule 39)

1. We, Bharat Biotech International Limited

   Genome Valley; Turkapalli; Shameerpet Mandal
   Rangareddy District; Hyderabad; Andhra Pradesh; India
   PIN:500 078
   An Indian Company

2. hereby declare that We are in possession of an invention titled **"A Novel Process for the Preparation and Purification of Recombinant Proteins"**

   (a) that the complete specification relating to this invention is filed with this application.
   (b) that there is no lawful ground of objection to the grant of a patent to us.

3. further declare that the inventors for the said invention are:

   (a) Dr. Vellimedu Kannappa Srinivas
   (b) Dr. Ella Krishnamurthy

   Adress of both the inventors is:
   Bharat Biotech International Ltimited
   Genome Valley, Turkapalli, Shameerpet Mandal
   Rangareddy district, Hyderabad; India
   Pin: 500 078

4 We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows: Not applicable.

5. We state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which We are the applicant/patentee: Not applicable

6. We state that the application is divided out of our application, the particulars of which are given below and pray that this application deemed to have been filed on ____________________ under section 16 of the Act. Not applicable.

Contd..Page.2.

7. That We are the assignee of the true and first inventors.

8. That our address for service in India is as follows:

Bharat Biotech International Limited
Plot No.265&266; Vamshisadan
Kamalapuri Colony Phase-II
Hyderabad-500 073

9. Following declaration was given by the inventors :
We the true and first inventors for this invention declare that the applicant herein is our assignee.

(Vellimedu Kannappa Srinivas)

(Ella Krishnamurthy)

10. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

11. Following are the attachments with the application:

(a) Complete specification (2 copies)
(b) Drawings (~~3 copies~~) - NIL
(c) Statement and Undertaking on Form-3
(d) Request for examination of application under Form-19.
Fee Rs. 6000/- in ~~Cash~~/Cheque/~~Bank Draft~~ bearing No. 770661 date 12.12.2003 on ICICI Bank.

We request that a patent may be granted to us for the said invention.

Dated this 24th day of December 2003

Signature:

Name : Pattipati Somasekhara Naidu

To
The Controller of Patents,
The Patent Office,
At Chennai.

FORM 2

PATENT ACT 1970

COMPLETE SPECIFICATION

1691/CHE/2003

30-12-2003.

Section 10

TITLE

A Novel Process for the Preparation and Purification of Recombinant Proteins

DUPLICATE

APPLICANT

BHARAT BIOTECH INTERNATIONAL LIMITED

GENOME VALLEY; TURKAPALLI

SHAMEERPET MANDAL; RANGAREDDY DISTRICT

HYDERABAD; ANDHRA PRADESH

INDIA

1691/CHE/2003

INDIAN LIMITED COMPANY

The following specification particularly described and ascertains the nature of the invention and the manner in which it is to be performed.

A Novel Process for the Preparation and Purification of Recombinant Proteins.

The present invention further relates to a novel process for the preparation and purification of viral antigenic proteins and other recombinant therapeutic proteins produced in either prokaryotic or eukaryotic cell systems.

BACKGROUND OF INVENTION

Use of prokaryotic and eukaryotic cell systems for the production of various therapeutic protein molecules is a common method in present day Biotechnology. In this process, the protein of interest is expressed in the said cell system by suitably engineering the molecular genetics of the expression system to incorporate a plasmid to promote the production of the desired proteins when suitably induced during the growth of the cells.

Similarly, the use of various cell substrates for the multiplication of viruses for the production of viral antigens is also a common practice. In this process, the cells are multiplied to large volumes and then they are "infected" with the required virus to facilitate the growth of the viruses. Alternately, transfected cells can also be grown. The viral harvests are obtained from the culture supernates or by cell lysis.

In both the cases as above, the proteins of interest is then concentrated , purified and further treated suitably(inactivated or cleaved) to prepare a therapeutic preparation or vaccine as the case may be.

The major challenges in any of the above processes are the following.

a) Recovery of the protein or antigen of interest in a most economic way.
b) Purification of the protein of interest to eliminate the contaminating substances like the host cell proteins, media components and any other materials used in the process.
c) Concentration of the purified protein to enable further processing.
d) Maintenance of the functional structure and activity of the protein during various stages of purification and the efficiency of recovery.
e) Preparation of a product of therapeutic value at the end of the process which shows equal or better performance as that of the reference product.

In order to achieve the above objectives, various processes are adapted. Recombinant molecules can be expressed as heterologous proteins in yeasts such as Sacharomyces cerevisiae, Pichia pastoris or E.coli and other organisms. Many biopharmaceuticals and other polypeptides such as Hepatitis B, Insulin, Streptokinase, Erythropoeitin, Human Growth hormone have been produced by recombinant DNA technology. The expressed proteins are purified from the culture of expression host to obtain the product. Similarly several viral vaccines are also produced by culture in different types of primary or continuous cell lines. The virus grown thus is then suitably purified, concentrated and inactivated/ or used as such for the preparation of vaccines.
Several steps of purification are generally adapted like clarification, centrifugation, filtration, and ultra-filtration, ammonium sulphate precipitation, use of silica beads,

continuous centrifugation , rate zonal gradient centrifugation, various methods of chromatography like gel permeation, size exclusion, affinity and Ion-exchange etc.

The purification processes named above have several draw-backs such as multiple steps, product loss, costly equipments and consumables and some times use of harmful chemicals like Cesium chloride etc and some of the processes make the product non-viable due to high cost of the "down stream process".

BRIEF DESCRIPTION OF THE INVENTION

According to the present invention as herein described, the recombinant proteins are made to be expressed in the vectors like E.coli, Yeast , Eukaryotic cell etc., extracted And purified by using HIMAX technology. It is to be understood that the word "HIMAX" is coined by us and refers to only the technology developed for this invention as explained here under.

OBJECTS OF THE INVENTION

1) The first object of the invention is to provide a method for the preparation and purification of recombinant proteins from the vectors by using HIMAX technology.
2) The second object of the invention is to prepare recombinant proteins which are highly purified without loss of biological activity.
3) The third object of the invention during the preparation of recombinant proteins is to achieve negligible interference of the nucleic acid or other contaminants if any.
4) The fourth object of the invention is to provide a process for simultaneous concentration and purification of various recombinant proteins, viral antigens, and biotherapeutic molecules.
5) The fifth object of the invention is to provide a process of protein purification which is less time consuming and cost effective.
6) Another embodiment of the invention is to provide a process of purification of live and inactivated viral antigens from cell lysate and fluid.
7) The seventh object of the invention is to purify the recombinant proteins by using divalent cations like Zn , Ca , Mg etc., in combination with anions like Acetate, Phosphate and chlorides.

DETAILED DESCRIPTION OF THE INVENTION

Now the details of the present invention:

a) The desired protein obtained through recombinant expression method or by culture in suitable tissue culture is obtained in a clarified harvest after various steps like cell lysis, cell debris removal and clarification etc.

b) A primary capture of the protein or antigen is carried out using the HIMAX method. Briefly the method involves using the addition of a divalent ionic salt

ranging from 0.2% to 10%, with counter ions of either phosphate, chlorides or acetate solution to form an insoluble matrix. The insoluble matrix thus obtained is then gently centrifuged to separate the bound antigen mass .The pellet thus obtained is then desorbed repeatedly with either Tris buffer of pH 8.0 to 8.5 or Tris buffer with EDTA at pH 7.0 to 8.0.

c) The desorbate containing the desired antigen is then further processed . In case of viral antigens , the process involved could be an inactivation followed by chromatography (ion exchange). In case of other antigens the desorbate is directly taken on to chromatography purification to obtain highly pure protein.

d) The final bulk product is obtained after pooling of the chromatographically purified fractions containing the desired proteins followed by diafiltration and or sterile filtration steps.

The above steps of invention are more clearly depicted in the following examples for some recombinant and cell culture proteins.

The examples provided herein are only for the explanation of the invention in detail and is not to be construed that the provided examples limits the scope of the alleged inventions.
Varying options which are within the scope of the invention but are not covered in the description that are available to the persons skilled in the art are to be taken as included in the present invention.

EXAMPLE I

Hepatitis B antigen production from a recombinant pathway

The cell lysate after fermentation is subjected to centrifugation and the insoluble fraction is treated with detergent. The supernatent after centrifugation was either subjected to Aerosil adsorption and desorption (traditional technology) (table 1) or to primary capturing of HBsAg by a batch procedure in which salts of divalent cations such as Calcium Magnesium and Zinc are added at 0.2% to 10% (w/v) in the presence of phosphates, Chlorides or Acetates to form white insoluble matrix. The insitu formation of the matrix further interact with the antigen and this process of protein capturing is referred as HIMAX technology. (tasble 2) This matrix was separated by centrifugation between 7000g to 10,000g and the bound antigen was desorbed repeatedly with this buffer of pH 8.5 .

The desorbate was further purified using an anion exchange matrix namely the DEAE.

The HbsAg activity in all the intermediate steps are given in table I and table II)
In another strategy the cell lysate is directly subkjected to primary capturing of the antigen by cations at 0.2% to 10% in the presence of phosphates, chlorides and acetates .
All subsequent steps were similar to earlier procedure.
The HBsAg activity in all the intermediate steps is given in table III

Flow Chart for HBs Ag production using HIMAX

Large Scale Fermentation

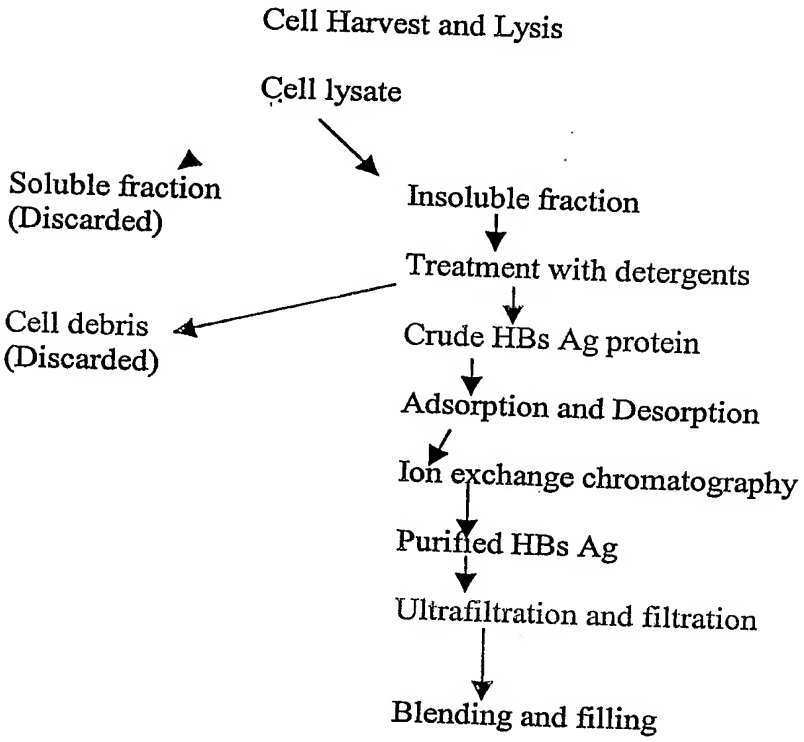

Table I Hepatitis B purification by traditional method

.S. No	Purification step	Activity (%)
1.	Total cell Lysate	100
2.	Soluble fraction	9
3.	In soluble fraction (membrane bound)	91
4.	Treatment with detergent	
5.	Centrifugation	
6.	Cell debris	16
7.	Supernatent (HBsAg protein)	34
8	Binding to Aerosil and desorption	20
9	Ion exchange chromatography.	15

Table II Hepatitis B purification by HIMAX method

.S. No	Purification step	Activity (%)
1.	Cell Lysate	100
2.	Soluble fraction	9
3.	In soluble fraction (HBsAg membrane bound)	91
4.	Treatment with detergent	
5.	Centrifugation	
6.	Supernatant (HBsAg protein)	84
7.	Adsorption and desorption	80
8	Ion exchange chromatography.	77

Table III Hepatitis B purification by HIMAX method

.S. No	Purification step	Activity (%)
1.	Cell Lysate	100
2.	Adsorption and Desorption	90
3	Ion Exchange chromatography	80

The major difference between table 2 and table 3 is the usage of detergent,
In the table 2, the insoluble fraction is treated with detergent, and further processing
Is carried with Adsorption and desorption technology.
While in the experiments represented in table 3, the cell lysate is directly
subjected to adsorption and desorption by HIMAX technology.

EXAMPLE II

Rabies antigen production from a cell culture pathway (FIG 2)

The large scale virus culture facilitates obtaining Rabies virus in the culture supernates. Traditionally the harvests of virus thus obtained are concentrated by ultrafilteration and then purified using the gradient ultracentrifugation on sucrose in a continuous or batch mode zonal centrifuge. In the present invention the culture supernatants are initially purified by the use of HIMAX for primary capturing of rabies antigen by a batch procedure in which salts of divalent cations such as Calcium Magnisium and Zinc are added to yield a final concentration of 8 to 10 fold (W/V) resulting in the formation of white insoluble matrix further interacts. The insitu formation of the matrix further interact with the antigen and this process of protein capuring is referred as HIMAX technology. This matrix was separated by centrifugation between 7000g to 10,000g and the bound antigen was desorbed repeatedly with tris EDTA buffer of pH 7.2.

The concentrated antigen so obtained is then inactivated by usual methods and further purified using an anion exchange matrix to obtain purified rabies antigen. The antigen is then diafiltered and blended as vaccine

The HIMAX purification yeilds with rabies antigen in all the intermediate steps are given in table IV.

Flow chart for HIMAX in Rabies Vaccine production

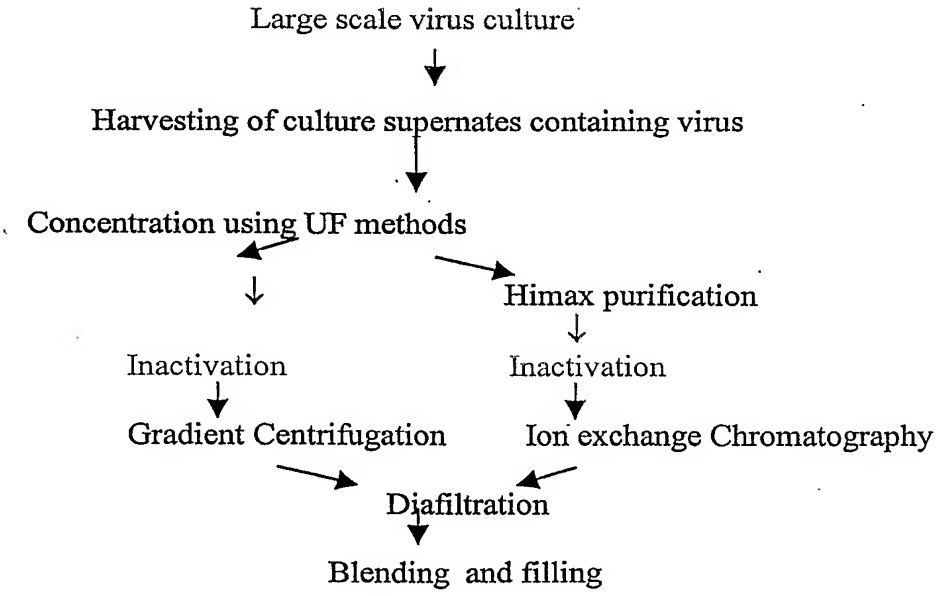

Table IV Rabies antigen purification by HIMAX

Sample lot no	Volume	HA activity per ml	Percent recovery
RAB Bulk 1-2003	1000 ml	1280	-
After HIMAX	120 ml	10240	96
RAB Bulk 2-2003	800 ml	2560	-
After HIMAX	95 ml	20480	95
RAB Bulk 3-2003	3000 ml	1280	-
After HIMAX	180 ml	20480	96

EXAMPLE III

Hepatitis A antigen production from a cell culture pathway

The large scale virus culture facilitates obtaining Hepatitis A virus in the culture as cell bound virus. Traditionally the harvests of virus are obtained as cell lysates which are clarified , inactivated and then purified using the gradient ultracentrifugation on sucrose in a continuous or batch mode zonal centrifuge. In the present invention the culture lysates are initially purified by the use of HIMAX for primary capturing of Hepatitis A antigen by a batch procedure in which salts of divalent cations such as Calcium Magnisium and Zinc are added to yield a final concentration of 8 to 10 fold (W/V) resulting in the formation of white insoluble matrix further interacts. The insitu formation of the matrix further interact with the antigen and this process of protein capuring is referred as HIMAX technology. This matrix was separated by centrifugation between 7000g to 10,000g and the bound antigen was desorbed repeatedly with tris EDTA buffer of pH 7.2.

The concentrated antigen so obtained is then inactivated by usual methods and further purified using an anion exchange matrix to obtain purified Hepatitis A antigen. The antigen is then diafiltered and blended as vaccine

The HIMAX purification yeilds with Hepatitis A antigen in all the intermediate steps are given in table V.

Flow chart for HIMAX in Hepatitis A production

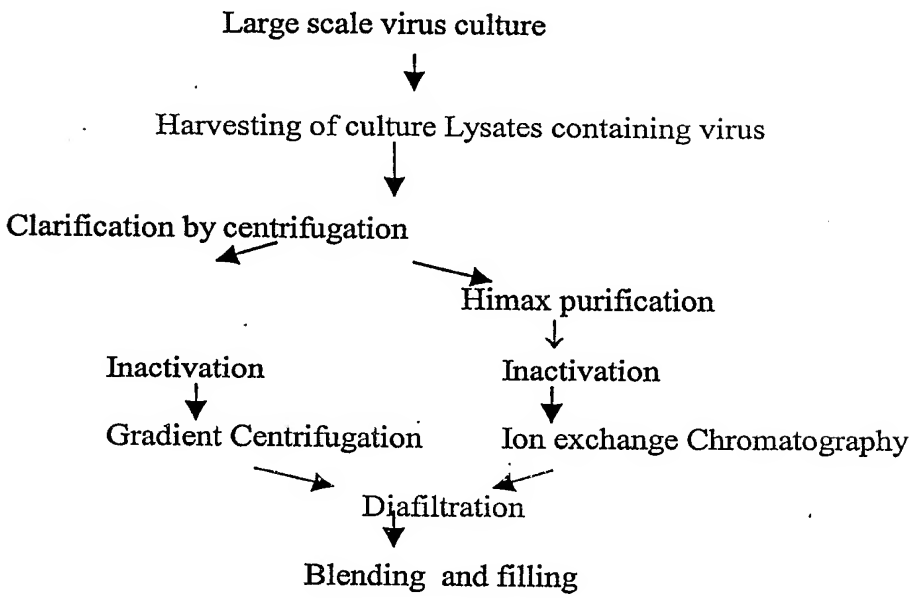

Table V -Hepatitis A antigen purification by HIMAX

Sample Lot No	Volume	ELISA units per ml	Recovery per cent
HAV Lot 2-03	100 ml	2560	
After HIMAX	9 ml	20480	72
HAV lot 3-03	150 ml	1280	
After HIMAX	16 ml	10120	84.3
HAV lot 4-03	90 ml	2560	
After HIMAX	90 ml	20480	88

CLAIMS :

1. A novel process for the purification of recombinant protein, expressed as either protein, or as particle or viral particle such as herein described, expressed in genetically engineering yeast, wherein the said yeast;

(a) Cells are subjected to lysis wherein the process of lysis is carried out in the absence of any detergent to obtain cell lysate:

(b) solution of step (a) is subjected to centrifugation at ranging from 1000 to 10,000g

(c) obtaining the solid of step (b) by decantation, wherein the solid contain the recombinant protein expressed as protein, particles or viral particles

(d) suspending the said solid in buffer ranging from pH 6 to 7.5 and optimally treating this, with a detergent such as herein described to solubulize the minute impurities.

(e) capturing the recombinant protein, or particle of step (d) with divalent ions like Zn, Ca, Mg in concentration ranging from 0.2% to 3%.

(f) recovering the said recombinant protein or particle with Tris buffer ranging from 0.1 to 1.5M, pH ranging from 8 to 8.5

(g) recovering the said protein through Ultrafiltration, Chromatography on colloidal silica and or ion exchange, hydrophobic and or affinity chromatography.

2. The process as claimed in claim 1, of step (d) , wherein the detergent is non ionic detergent,

3. The process wherein the steps of Ultrafiltration is carried out using membrane filters of 100-300K molecular weight cut off

4. The process as claimed in claim 1, the solid obtained in step (c) is treated with divalent ions without treating with detergent

5. The process wherein the ion exchange matrices are selected from anionic exchange resins such as sulphated cellulose/DEAE matrics.

6. A novel process for the purification of the recombinant protein, protein expressed as particle or viral particle.

Dated this 24th day of December 2003

P.Somasekhara Naidu
Authorised Signitory of the Applicant

ABSTRACT

A novel process for the purification of recombinant protein expressed as protein or particle is herewith described.

In this purification process, the protein is purified by hydrophobic interaction. The interaction of this protein step resulted in an increase in recovery and purity from 15%-80%. The protein further purified has its application in vaccines and pharmaceuticals.